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Thermally Regulated Translational Control of FRQ Mediates Aspects of Temperature Responses in the Neurospora Circadian Clock

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Summary

Two forms of FRQ, a central component of the Neurospora circadian clock, arise through alternative in-frame initiation of translation. Either form alone suffices for a functional clock at some temperatures, but both are always necessary for robust rhythmicity. Temperature regulates the ratio of FRQ forms by favoring different initiation codons at different temperatures; when either initiation codon is eliminated, the temperature range permissive for rhythmicity is demonstrably reduced. This temperature-influenced choice of translation-initiation site represents a novel adaptive mechanism that extends the physiological temperature range over which clocks function. Additionally, a temperature-dependent threshold level of FRQ is required to establish the feedback loop comprising the oscillator. These data may explain how temperature limits permissive for rhythmicity are established, thus providing a molecular understanding for a basic characteristic of circadian clocks.

Introduction

Circadian rhythms are daily (~24 hr) endogenous oscillations that are a fundamental aspect of control found in virtually all groups of organisms (Bünning, 1973; Liu et al., 1995; Dunlap, 1996). Clock genes have been identified in several organisms (Konopka and Benzer, 1971; Feldman and Hoyle, 1973; Kondo et al., 1994; Sehgal et al., 1994; Vitaterna et al., 1994; Millar et al., 1995), and in the last decade rapid progress has been made in a few model systems. However, only the *frequency* (*frq*) gene in Neurospora and *per* and *tim* genes in Drosophila can be said with some confidence to be bona fide components of circadian oscillators (Aronson et al., 1994a; Hall, 1995; Sehgal, 1995).

A variety of environmental stimuli, including light, temperature, and chemicals, have been shown to affect circadian clocks. Of these, temperature is especially interesting, since unlike light and other stimuli that act exclusively as entrainment signals, temperature can affect circadian clocks in three different ways. First, the period length of rhythms remains about same over a broad temperature range—a mechanism commonly called ''temperature compensation'' that allows the clock to make accurate time measurements despite large variations in ambient temperatures in the natural environment (Hastings and Sweeney, 1957; Pittendrigh, 1993). Second, both temperature pulses and temperature steps can reset the oscillator (Zimmerman et al., 1968; Francis and Sargent, 1979; Edery et al., 1994; Gooch et al., 1994; for review, see Sweeney and Hastings, 1960) so that temperature stands with light in importance as an entrainment factor for clocks. Although temperature responses have been extensively studied physiologically, they are not yet understood at the molecular level.

The third and least understood aspect of circadian temperature influences is anchored in the observation that there are physiological temperature limits for circadian rhythmicity; i.e., clocks can only function within a restricted range of temperatures. Outside this limit, the clock will stop running and freeze at a certain phase from which rhythmicity resumes upon a return to permissive temperatures, a property well known in the classical circadian literature (see Discussion). A universal and critically informative aspect of this phenomenon is that the physiological limits for rhythmicity do not coincide with the temperature range permissive for growth but instead lie well within these limits. We reasoned that an ideally adapted biological clock would function over the entire physiological growth range, and hence this universally observed restriction in the range permissive for rhythmicity very likely reflected a limit in the capacity of the oscillator mechanism itself to adapt to temperature extremes. If indeed this were so, then it should be possible to understand how the physiological limits for rhythmicity are set by understanding how clock components are regulated by temperature. Our current understanding of this molecular mechanism of the circadian oscillator makes it possible to address this question at the molecular level.

frq mRNA and FRQ protein are the central components of the Neurospora circadian clock (Dunlap, 1993, 1996). The circadian oscillator is comprised of an autoregulatory feedback loop in which the amount of *frq* transcript is depressed by its product, FRQ protein (Aronson et al., 1994a). Both the level of *frq* message and FRQ protein oscillate with a period that is the same as the overt rhythm in development exhibited by the organism (Aronson et al., 1994a; Garceau et al., 1997 [this issue of *Cell*]). Additionally, *frq* regulates at least indirectly genes such as the clock-controlled genes (ccgs) that are transcriptionally regulated (Loros et al., 1989; Bell-Pedersen et al., 1996a), and other activities required for the overt expression of the rhythm. Constitutive overexpression of *frq*, established by fusing *frq* to an inducible promoter, abolishes the rhythm and resets the phase of the clock (Aronson et al., 1994a). Disruption of the *frq* locus abolishes normal rhythmicity (Aronson et al., 1994b). Light resets the clock by rapidly increasing the level of *frq* message and FRQ protein, an effect that is independent of the feedback loop (Crosthwaite et al., 1995).

frq transcripts ca. 4.5 kb in length have been predicted to encode a putative 989 aa protein (Aronson et al., 1994b). However, recent data have revealed that there are two FRQ forms, $FRQ^{1–989}$ and $FRQ^{100–989}$, expressed as a result of alternative initiation of FRQ translation from two of three in-frame initiation codons (Garceau et al., 1997). As there are many examples of eukaryotic

mRNAs using alternative in-frame initiation to produce proteins having different properties (Descombes and Schibler, 1991; Geballe, 1996), this raises the possibility that the two forms of FRQ may have different functions. *frq* mRNA contains a very long 5′ untranslated region (5′ UTR) ca. 1.5 kb in length with 6 short open reading frames (uORFs) that precede the FRQ ORF. Together, these data suggest that a translational control mechanism could be involved in the regulation of FRQ expression, and this regulation might be important for operation of the clock under certain conditions.

In this study, we have analyzed the function and regulation of the two FRQ forms. We present evidence consistent with a translational control mechanism in which alternative initiation, in direct response to ambient temperature, directs the synthesis of two similar but functionally distinct forms of the clock protein FRQ. High temperature favors the translation of a long form that is required for rhythmicity at temperatures near to the high end of the physiological range. Conversely, low temperature favors the translation of a shorter form required at the low end of the physiological range, temperatures at which an equivalent amount of long FRQ will not substitute. This novel adaptive mechanism allows Neurospora to keep its clock running over a wide range of temperatures. Our data also suggest that there is a threshold level of FRQ required by the clock and that this threshold level increases with increasing temperature. Taken together, these data may serve to illuminate the molecular basis for a general characteristic of all circadian clocks; i.e., how the temperature limits permissive for rhythmicity are established and maintained.

Results

Either Form of FRQ Is Able to Support Circadian Rhythmicity

There are three methionine codons (AUG) located within the first 100 codons of the *frq* ORF: AUG#1 (codon #1), AUG#2 (codon #11), and AUG#3 (codon #100). As previously shown, *frq* mRNA translation can be initiated from at least two of these AUGs to generate two forms of FRQ: $FRQ^{1–989}$ and $FRQ^{100–989}$ (Garceau et al., 1997). To identify functional distinctions between the two forms of FRQ, constructs were made that would support the translation of only the long form, $FRQ^{1–989}$ (pJC101-L), only the short form, $FRQ^{100–989}$ (pYL34-S and pYL15-S), or neither form (pYL31-N) (Figure 1A; Garceau et al., 1997). These constructs were targeted to the *his-3* locus of frq^{10}, an *frq*-null strain (Aronson et al., 1994b), and the resulting transformants examined for rhythmicity at 25°C (Figure 1B). Each form of FRQ alone is able to rescue the conidiation banding rhythm of frq^{10}. The period of the pJC101-L transformants (only $FRQ^{1–989}$) is slightly shorter than that of pKAJ120 transformants (Table 1), while the period of the pYL15-S and pYL34-S transformants (only $FRQ^{100–989}$) is longer (Table 1); as expected, pYL31-N failed to rescue the rhythm. The daily bands of conidiation are broader in strains transformed with pJC101-L, pYL15-S, and pYL34-S, suggesting a change in the amplitude or quality of circadian control in these strains as compared to wild-type transformants.

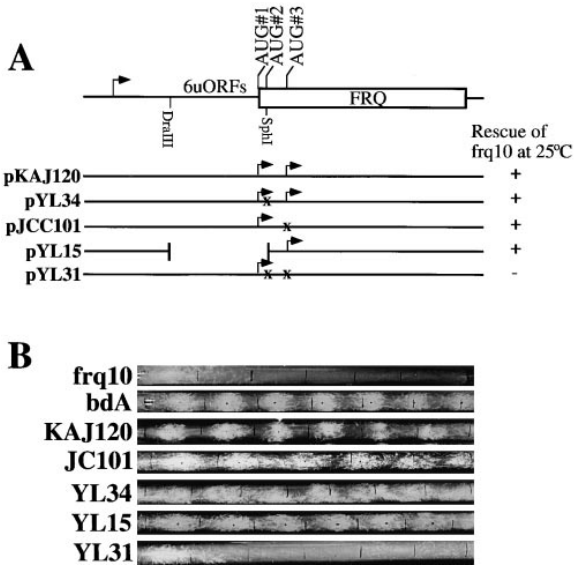

Figure 1. Either Form of FRQ Is Sufficient to Support a Functional Clock

(A) Schematic diagram of the *frq* locus and constructs selectively eliminating AUG codons. Top: the arrow represents the transcriptional start site for the *frq* mRNA. The box labeled "FRQ" represents the complete ORF with the positions of internal AUGs marked. Bottom: various AUG deletion constructs (see Experimental Procedures). Arrows represent AUG#1 and AUG#3, and "X" designates a mutation eliminating one of the AUGs.

(B) Race tube analyses showing rhythmicity in transformants bearing various AUG-deletion constructs at 25°C. Conidiospores were inoculated at left end of each race tube as shown, cultures were grown in light for one day and then transferred into constant darkness to set the clock to subjective dusk, and the position of the growth front marked (vertical lines) immediately and at 24 hr intervals thereafter. Dots mark the center of each clock-controlled conidial band. No rhythm is apparent in frq^{10} or in frq^{10} strains transformed with YL31.

This is especially true for pYL34-S transformants for which it is sometimes difficult to locate the center of the bands. These data indicate that either form of FRQ is capable of supporting rhythmicity at 25°C but suggest that both forms of FRQ may be required to generate normal high amplitude rhythms.

The Physiological Temperature Limits of the Clock Are Differentially Restricted When Only One Form of FRQ Is Expressed

Having shown that under some growth conditions either form of FRQ is sufficient for rhythmicity, we were anxious

Table 1. Conidiation Banding Rhythms of Different Strains

	Period (hr) + SD			
Strains	KAJ120	YL34	JC101	YL15
Temperature				
18°C	23.1 + 1.2	26.6 + 3.0	ND	23.9 + 2.3
20°C	23.9 + 1.1	25.8 + 1.4	ND	24.1 + 1.0
22°C	23.0 + 0.9	26.9 + 1.5	21.6 + 0.9	24.1 + 1.4
25°C	22.9 + 0.6	25.3 + 1.1	21.8 + 1.4	23.5 + 1.2
27°C	21.5 + 0.8	ND	21.6 + 1.0	22.5 + 1.3
30°C	20.7 + 0.5	ND	21.5 + 0.6	22.7 + 1.2

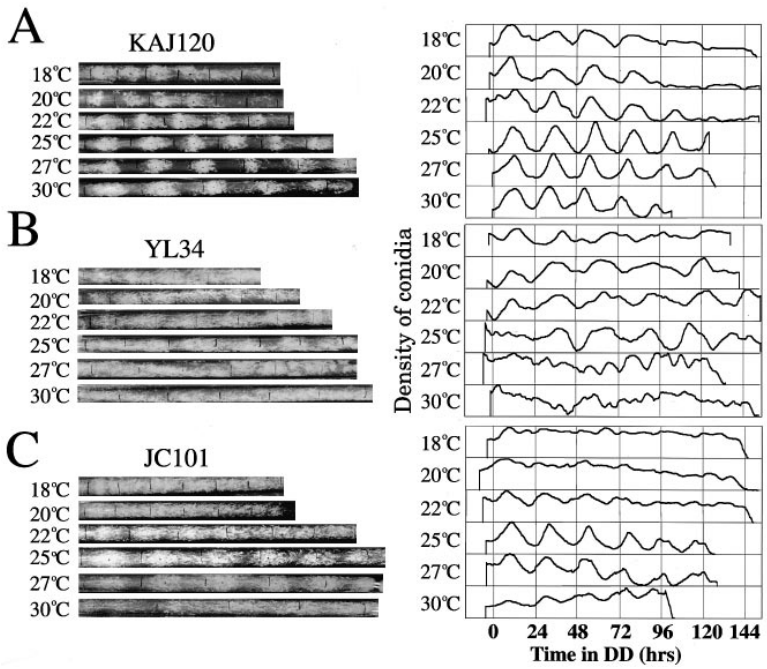

Figure 2. Mutation of Initiation Codons within the FRQ ORF Reduces the Temperature Range Permissive for Rhythmicity

Race tube data are shown on the left, and the densitometric analysis of the images is shown on the right (see Experimental Procedures).

(A) Transformants bearing the intact *frq* locus (KAJ120) exhibit normal rhythmicity at all temperatures across the physiological range.

(B) Deletion of AUG#1 in YL34-S eliminates overt rhythmicity at temperatures near the high end of the physiological temperature range.

(C) Mutation of AUG#3 in JC101-L transformants selectively eliminates overt rhythmicity at temperatures near the low end of the physiological temperature range.

to know why both forms are necessary. We reasoned that this alternative initiation of FRQ plus the existence of the long 5′ UTR in *frq* mRNA might suggest that the choice of initiation codon is regulated by a translational control mechanism that could adapt the expression of FRQ for operation of the clock under different environmental conditions. For instance, uORF studies in other eukaryotic genes (e.g., *GCN4* in yeast or *arg-2* in Neurospora) (Hinnebusch, 1996; Luo and Sachs, 1996) have shown that nutritional regulation can affect translational control; it is known that glucose levels in the medium dictate the level of *frq* expression (Aronson et al., 1994a) and that histidine auxotrophy or supplementation can affect period length of the Neurospora clock (Sargent and Kaltenborn, 1972). Alternatively, the Neurospora circadian clock (like other clocks) has physiological temperature limits (ca. 15°C to 34°C) (Francis and Sargent, 1979). Therefore, to evaluate the biological significance of—and the functional distinction between—the two forms of FRQ, we examined the clock under different environmental conditions in strains expressing only one of the two forms of FRQ.

Rhythmicity was evaluated on race tubes in YL34-S (ΔATG#1), JC101-L (ΔATG#3), and KAJ120 (*frq*$^{+}$) at temperatures between 18°C and 30°C (Figures 2 and 3, Table 1) and under different nutritional conditions (data not shown). The data suggest that the two forms of FRQ play no apparent role in nutritional effects on the clock but are of central importance in defining the physiological temperature limits for rhythmicity. In control experiments (Figure 2A), KAJ120 showed clear rhythmicity at all temperatures tested, although the amplitude of the rhythm became reduced at both temperature extremes. In a similar manner, YL34-S retained rhythmicity at the lower temperatures (albeit with the characteristic lower amplitude), but surprisingly, the rhythm deteriorated at 27°C and cultures became completely arrhythmic at 30°C (Figure 2B). In contrast, JC101-L (ΔATG#3) displayed a reciprocal defect in which it retained rhythmicity at the high end of the normal physiological temperature range but became arrhythmic at temperatures 20°C and below (Figure 2C). Although period lengths of the rhythms are difficult to determine in some cases because of the low amplitude, the temperature compensation of the period does not appear to be affected within the temperature range permissive for rhythmicity (Table 1).

Temperature Determines the Absolute Level of, and Ratio between, the Two FRQ Proteins

These results clearly indicate that without either form of FRQ, the temperature limit for rhythmicity is significantly restricted from one end of the normal temperature range. The distinct symmetry of the results—loss of low temperature rhythmicity correlated with loss of FRQ$^{100–989}$ and loss of high temperature rhythmicity with loss of FRQ$^{1–989}$—prompted us to evaluate the effect of ambient temperature on the normal pattern of expression of the FRQ isoforms (Figure 3). Samples for analysis in Western blots were collected at the peak of the FRQ expression, DD18 (CT8); the two groups of bands (indicated by arrows) correspond to the two forms of FRQ initiated from two AUGs (Garceau et al., 1997), and the multiple bands of different mobility within each group are due to different phosphorylation stages of FRQ (Garceau et al., 1997). As shown in Figure 3A, temperature clearly dictates the expression profile of the FRQ isoforms: although both forms of FRQ are present at all temperatures examined, the ratio of FRQ$^{1–989}$/FRQ$^{100–989}$ increases as the temperature increases (Figure 3C). While at low temperature (18°C) the amount of FRQ$^{100–989}$ is similar to or slightly more than that of FRQ$^{1–989}$, at any warmer temperatures full-length FRQ becomes the clearly dominant form. In addition to changing this ratio, the overall

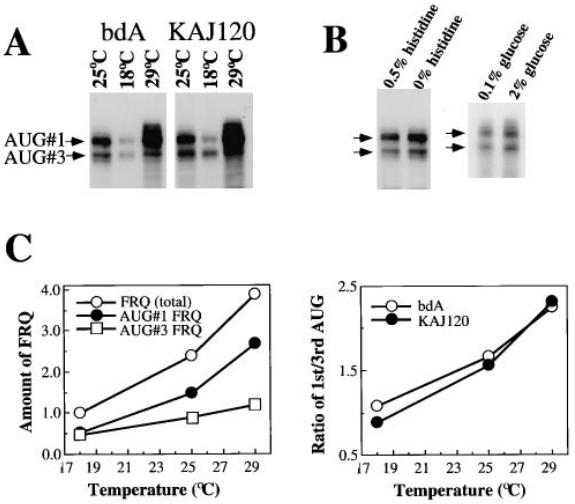

Figure 3. Ambient Temperature but Not Nutrition Determines both the Absolute Level of FRQ Expression and the Relative Amount of the Two FRQ Forms

(A) Western blots showing that FRQ expression in both wild-type (*bdA*) and KAJ120 (*frq*$^{+}$) transformants increases with temperature. The two arrows point to the FRQ products initiated from AUG#1 (FRQ$^{1–989}$) and AUG#3 (FRQ$^{100–989}$), respectively.

(B) Western blots showing that FRQ expression is not substantially affected by changes in histidine or glucose supplementation.

(C) Densitometry analysis of the FRQ expression profiles shown in (A). Left panel: although the absolute level of each form of FRQ increases with temperature, the increase of FRQ$^{1–989}$ is much greater than that of FRQ$^{100–989}$. Reflecting this (right panel), the ratio of FRQ$^{1–989}$/FRQ$^{100–989}$ increases with temperature.

level of FRQ also increased about 4× from 18°C to 29°C (Figures 3A and 3C), largely owing to enhanced translation of full-length FRQ (Figure 3C). In contrast to the temperature effect, but in confirmation of the results of physiological analyses, manipulation of histidine and glucose concentration did not affect the expression profile of FRQ (Figure 3B). Overall, these data clearly demonstrate that the alternative initiation of FRQ is regulated as a function of ambient temperature within the physiological range.

Temperature-Dependent Synthesis of Multiple Forms of FRQ Reflects both Quantitative and Qualitative Requirements for FRQ by the Clock

In the aggregate, the physiological data mesh nicely with the observed temperature dependence of translation initiation and reveal a novel adaptive mechanism that enables the organism to extend the physiological range over which the clock will keep time. We noted, however, that the quality of the rhythms of YL34-S and JC101-L are inferior to those observed in wild-type strains. This suggests that an optimal clock may still require both FRQ forms to act together at all temperatures. Alternatively, the deterioration of rhythmicity associated with loss of one of the FRQ forms may simply reflect quantitative rather than qualitative requirements; i.e., perhaps more FRQ is needed at higher temperatures, and the observed temperature-dependent translation-initiation mechanism evolved simply as a response to this need.

To evaluate these alternatives, we examined the FRQ

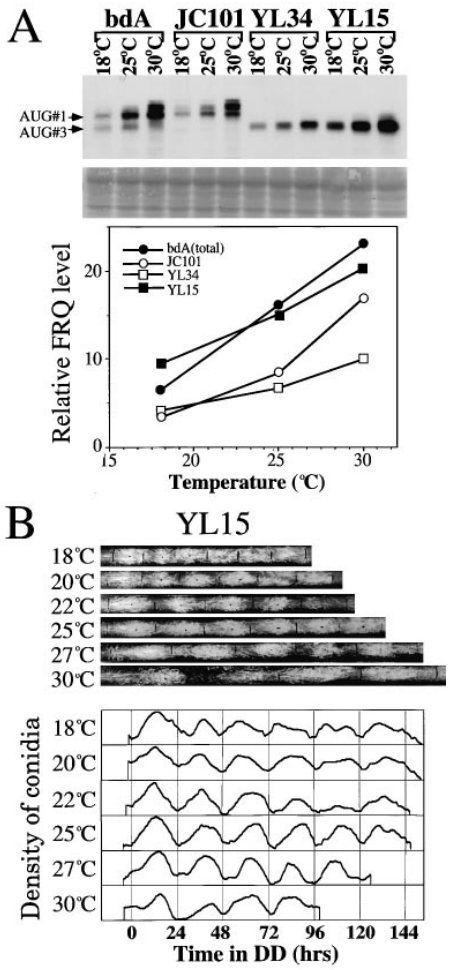

Figure 4. Deletion of AUG#1 Results in Enhanced Expression of FRQ from AUG#3 That Can Satisfy the Quantitative Requirement of the Clock for FRQ

(A) Western blot showing FRQ expression as a function of temperature in strains bearing deletions in different AUGs. The arrows mark the positions of the minimally phosphorylated forms of FRQ arising from AUG#1 and AUG#3. Below this is shown the same membrane stained with amido black to verify protein loading. At the bottom, densitometric analysis of the Western blots shows that expression of FRQ increases with temperature, and that elimination of part of the 5′ UTR and AUG#1 (in YL15-S) boosts expression from AUG#3 to levels comparable to that of wild type.

(B) Top: race tubes showing that rhythmicity is restored at all temperatures when deletion of the uORFs and AUG#1 boosts expression of FRQ$^{100–989}$ in strain YL15-S. Bottom: densitometric analysis of the images above confirms that both period and phase of the rhythm are relatively constant despite changes in temperature and growth rate.

expression profile at different temperatures in these and in additional mutant strains (Figure 4A). As expected, the form of FRQ expressed depends on which AUG is present, and otherwise the total amount of FRQ increases as the temperature increases. However, the slope of the increase is different for the two forms: the increase of FRQ$^{100–989}$ (YL34-S) with temperature is much slower than that of FRQ$^{1–989}$ (JC101-L) as predicted based on the profiles of the two forms in wild-type *frq*. The data are thus consistent with an overall increase in

the amount of translation with temperature, but with a ribosome-scanning mechanism determining the choice of AUG such that as the temperature decreases, the first AUG is missed more often (see Discussion). To evaluate this, FRQ expression and overt rhythmicity were monitored in strain YL15-S, which bears an extended deletion covering much of the *frq* 5′ UTR and extending into the ORF past the first and second AUG. As expected, this strain makes only $FRQ^{100–989}$ and, consistent with a ribosome-scanning mechanism, FRQ is made at a level comparable to that seen in frq^{+}. Thus, in quantitative terms, the total amount of FRQ in JC101-L and YL34-S is less than that in frq^{+}, especially at high temperature for YL34-S and at low temperature for JC101-L. This suggests that the loss of rhythmicity in these two strains at temperature extremes may indeed be due to insufficient FRQ expression, and the low level of FRQ in YL34-S at all temperatures may explain the very low amplitude of its rhythm even at permissive temperatures. Consistent with a quantitative requirement for FRQ, YL15-S produces a total level of FRQ at all temperatures comparable to that seen in wild type and is capable of at least rudimentary rhythmicity at all temperatures (Figure 4B). Even at temperatures where YL34-S and JC101-L are arrhythmic, substantial FRQ is expressed (JC101-L at 18°C and YL34-S at 30°C compared to frq^{+} [*bdA*] at 18°C and 30°C); this suggests that the Neurospora clock requires a threshold level of FRQ before the clock feedback loop can function and that this threshold amount is higher at higher temperatures.

A model postulating the requirement of a simple temperature-dependent threshold level of required FRQ cannot account for all of the data, however. First, the amount of $FRQ^{1–989}$ in JC101-L is comparable to that of $FRQ^{100–989}$ in YL34-S at 18°C (Figure 4A), and yet JC101-L is arrhythmic at that temperature, whereas YL34-S is rhythmic. This suggests that $FRQ^{100–989}$ is qualitatively different and may function better than $FRQ^{1–989}$ at low temperature. Similarly, although YL15-S produces total levels of FRQ (but only $FRQ^{100–989}$) comparable to wild type and is capable of rhythmicity at all temperatures tested, the rhythm is generally of a lower amplitude than that seen in wild type, especially at 30°C (Figures 2 and 4B), which suggests that the short FRQ form does not function well at high temperature. The poor quality of the rhythm in JC101-L at 30°C also suggests that long FRQ form alone cannot fully accomplish the job. These data indicate qualitative differences between the two forms of FRQ and establish that an optimally adjusted circadian clock will require both forms.

Thermal Regulation of FRQ Isoform Production Is Controlled by Translational Initiation

The results presented in Figures 3 and 4 indicate that the ratio of $FRQ^{1–989}$/$FRQ^{100–989}$ increases with temperature, but our assertion that this change reflects choice of translational initiation site is subject to the caveat that we have examined only total FRQ levels. Similar data could also arise as a result of distinctly different rates of turnover for the two FRQ forms if, for instance, the larger FRQ was more stable at higher temperatures and the smaller FRQ more stable at low temperatures.

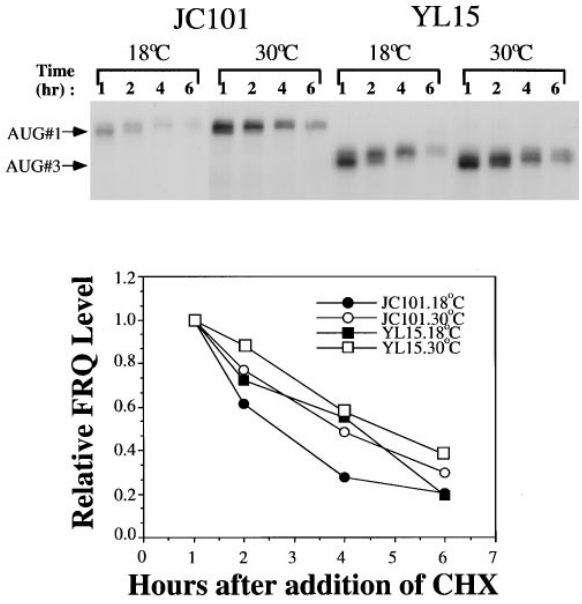

Figure 5. Temperature-Dependent Differences in the Amounts of the Two Forms of FRQ Are Not the Result of Differences in Protein Stability

Strains JC101-L and YL15-S, each of which only express one form of FRQ, were used. Liquid cultures were grown in constant light for more than 18 hr prior to the addition of cycloheximide at 10 μg/ml. Top panel shows the results of Western blot analysis, with the corresponding densitometry in the bottom panel.

We therefore compared the stability of the two forms of FRQ at different temperatures. Strains were used that produce only the large (JC101-L) or the small (YL15-S) forms of FRQ. FRQ is processively phosphorylated (signaled by a shift to slower mobility) and eventually degraded in its most phosphorylated form at the end of the night. These and other data suggest that such phosphorylation could be a factor in influencing the rate of turnover of FRQ (Garceau et al., 1997). If so, in these experiments the instantaneous rate of turnover might be changing with time, so that the distinctions between stabilities of the forms of FRQ that we would like to measure could be lost. To eliminate this uncertainty associated with changing phosphorylation of FRQ, we performed the experiment in constant light, where the level of FRQ is high and it is both highly and stably phosphorylated (N. Y. G. et al., unpublished data). De novo synthesis of FRQ was suspended by the addition of 10 μg/ml cycloheximide to the culture (sufficient to inhibit greater than 95% of the protein synthesis in Neurospora) (Dunlap and Feldman, 1988). Following the addition of the drug, the cycle of phosphorylation continues, and the amount of either form of FRQ decreases at a rate primarily reflecting only the temperature and not the form of FRQ (Figure 5, compare YL15-S at 18°C to JC101-L at 18°C and YL15-S at 30°C to JC101-L at 30°C). This result is consistent with our previous data describing FRQ levels as a function of temperature (Figures 3 and 4) and confirms that the differences we have seen in the amount of $FRQ^{1–989}$ versus $FRQ^{100–989}$ reflect differences in translation initiation rather than protein stability. This modulation of FRQ levels by temperature

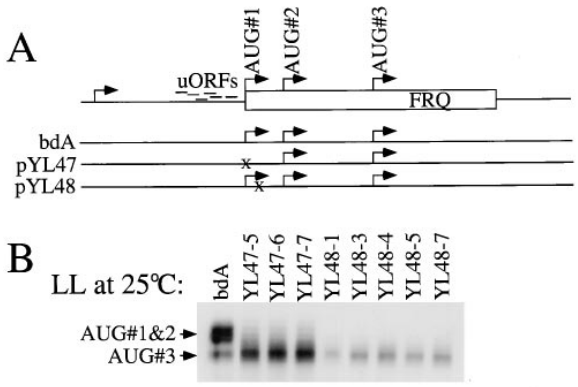

Figure 6. AUG#2 Is Not Used to Initiate FRQ Production under Normal Conditions

(A) Schematic diagram of *frq* locus and AUG deletion constructs. Top: the arrow on the left represents the transcriptional initiation site for *frq* mRNA, and six short bars in the 5′ UTR represent the six short uORFs. The positions of the three in-frame FRQ AUGs are also labeled. Below this are shown the intact *frq* locus and two mutation constructs. In pYL47, "X" represents the mutation of AUG#1 to AUU; in pYL48, it represents the 1 nt deletion introduced between AUG#1 and AUG#2.

(B) The expression profile of FRQ in YL47 and YL48 transformants under constant light shows that AUG#2 is only rarely used even in the absence of AUG#1 and is never used when AUG#1 is intact. Three YL47 transformants and five YL48 transformants are shown.

must be mediated by a factor(s) that is sensitive to small changes in the ambient temperature and that acts to influence the choice of FRQ initiation codons.

AUG#2 Is Not Used to Initiate FRQ Expression under Normal Conditions

Although the sequence data show that FRQ AUG#2 lies in a very poor translation-initiation context, it could still be used. To clarify this, two constructs were made: pYL47, which has a 1 nt mutation to eliminate AUG#1, and pYL48 bearing a 1 nt deletion between AUG#1 and AUG#2, placing AUG#1 out of frame with the FRQ ORF (Figure 6A). Both constructs were introduced into *frq*^{10}, and the FRQ expression profile of the transformants was examined under constant light (LL) (Figure 6B). In pYL48 transformants, no FRQ initiated from AUG#2 was detected, suggesting that AUG#2 is not used under normal translation conditions. In pYL47 transformants, however, while most FRQ is initiated from AUG#3, a small amount of FRQ initiated from AUG#2 can be seen (Figure 6B), suggesting that AUG#2 can be used very inefficiently when AUG#1 is not there. In addition, the level of FRQ in pYL47 transformants is much higher than that of pYL48 transformants. These data and those in Figure 4 are consistent with use of a ribosome-scanning mechanism for choice of FRQ initiation site, in which ribosomes scan beginning upstream of AUG#1 and the ribosomes that miss AUG#1 will then continue downstream to initiate from AUG#3. If this is the case, ambient temperature must then influence the efficiency of ribosome initiation from AUG#1 in order to regulate choices of AUGs: as temperature decreases, more ribosomes will miss AUG#1.

Discussion

The *frq* gene encodes central components of the Neurospora circadian oscillator, producing transcripts that yield two forms of the FRQ protein as the result of alternative initiation of translation (Garceau et al., 1997). Either form alone is sufficient to complete a functional clock at some temperatures, but under other less permissive conditions strains lacking one form are arrhythmic, and furthermore, under all conditions such clocks never appear as robust as wild type. Temperature regulates the ratio of the two FRQ forms by favoring different initiation codons at different temperatures, and when either of the initiation codons is eliminated, the temperature range permissible for rhythmicity is clearly reduced. This temperature-influenced choice of translation-initiation site represents a novel adaptive mechanism that extends the physiological temperature range over which the clock can function. Our data further indicate that there is a threshold level of FRQ required to establish the feedback loop comprising the oscillator and that this threshold level increases as the temperature increases. Altogether, these data describe several aspects of the temperature adaptations of circadian oscillators not anticipated by previous work.

Temperature and Circadian Oscillators

In addition to light, temperature is the other major environmental factor known universally to influence all circadian oscillators. Three facets of circadian temperature responses have been described physiologically—temperature limits for rhythmicity, temperature resetting, and temperature compensation—and insights into all three are explicit or implicit in the data presented here.

The least understood aspect of circadian temperature influences, and the one for which these data potentially provide the greatest insight, is the mechanism through which the temperature limits permissive for clock function are established. Although variously described under different names ("secondary arrhythmicity" [Zimmerman et al., 1968] or "holding" [Pittendrigh, 1974] in Drosophila, "conditional arrhythmicity" in Gonyaulax [Njus et al., 1977], "stopping" in Neurospora [Francis and Sargent, 1979]), the phenomenon is well characterized in the classical circadian literature beginning as early as 1934 (Kalmus, 1934) (reviewed in Sweeney and Hastings, 1960; Bünning, 1973) and continuing into the modern era (Martino-Catt and Ort, 1992). Based on the work reported here, this mechanism can be now be understood in terms of (i) the existence of a minimum required threshold concentration for one or more of the central clock components such as FRQ, a level that increases with increasing temperature, and (ii) an adaptive mechanism whereby levels sufficient to achieve that threshold (but not too high) can be maintained over a wide range of temperatures. In Neurospora, the existence of this minimum level can be seen by comparing Figure 2, where insufficient expression of FRQ$^{100–989}$ in YL34-S at high temperatures results in arrhythmicity, with Figure 4B, where the loss of rhythmicity is reversed by increased expression of the same FRQ$^{100–989}$ protein in YL15-S. The adaptive mechanism that maintains the required threshold of FRQ across a wide temperature range involves

temperature-dependent choice of translation-initiation sites combined with the naturally increased rate of protein synthesis at higher temperatures. While other organisms may have evolved different adaptive mechanisms, this general rule—temperature limits for rhythmicity are defined by the mechanism used to achieve minimum levels of a central clock component—may be similar in many circadian systems.

Another universal aspect of circadian temperature responses illuminated by these data is temperature-induced resetting of the oscillator. In all circadian clocks, temperature steps will reset the clock, and temperature cycles with amplitudes of as little as 2°C are sufficient to entrain the rhythm (Francis and Sargent, 1979). As shown by Figures 3 and 4, FRQ levels increase dramatically with temperature, a response qualitatively similar to that seen with *frq* transcript (Crosthwaite et al., 1995) and FRQ (N. Y. G. et al., unpublished data) following light signals sufficient to reset the clock. Although presently nothing is known about the kinetics of this temperature response with respect to the kinetics of resetting, these data do suggest that temperature may entrain circadian oscillators by acting to change the level of a central component of the clock such as FRQ.

Temperature compensation is a third fundamental aspect of circadian temperature responses (Pittendrigh, 1993) to which the present data speak. A variety of models have been proposed to explain compensation (e.g., Hastings and Sweeney, 1957; Njus et al., 1974; Huang et al., 1995), none firmly supported by the data (reviewed in Dunlap, 1996), but an amplitude model (Lakin-Thomas et al., 1991) may be of interest in the present context. This model posits that the amplitude of the oscillator (the size of the limit cycle describing the oscillation) increases with temperature, as does the linear velocity around the cycle, so that the overall cycle length remains temperature compensated. Although an actual increase in the amplitude of the oscillator, or of a state variable of an oscillator, with temperature has never been shown, we found the level of FRQ at the peak of the FRQ oscillation to increase with temperature (Figures 3 and 4). This would be consistent with the temperature-dependent increase in the amplitude of the oscillation predicted by the model. More generally, however, whether or not the details of any model are supported, one thing is clear: temperature compensation in Neurospora is not achieved by maintaining a constant level of oscillator state variables but rather by a dynamic process that incorporates a substantial temperature-dependent change in the natural level of the state variables. In this view, mutations that alter the stability of a clock component or that interfere with interactions that influence the stability of a component (such as per^{L} in Drosophila; Huang et al., 1995) would appear to have altered temperature compensation.

Why Does the Neurospora Clock Need Two FRQ Forms?

Alternative translational initiation can greatly amplify the potential functions of a gene because the alternative forms of the protein so produced may have cooperative, alternative, or even opposing effects (reviewed in Geballe, 1996). For instance, alternative initiation determines the location and properties of human fibroblast growth factor 2 (Vagner et al., 1995) and dictates whether transcriptional-activator or -inhibitor proteins are made from the same transcript in liver (Descombes and Schibler, 1991). For FRQ, the answer to the above question has both quantitative and qualitative aspects.

Quantitatively, a temperature-dependent threshold amount of FRQ appears required to complete the feedback cycle that constitutes the clock. At 18°C and 20°C, insufficient FRQ (full-length) is made in JC101-L to support a rhythm, but because of the steep change in the amount of full-length FRQ with temperature, at 25°C and 30°C the clock functions with only full-length FRQ (Figures 2 and 4). Conversely, at elevated temperatures in YL34-S the rhythm deteriorates because insufficient FRQ ($FRQ^{100–989}$) is made (Figure 2), and yet when translational controls are eliminated to allow increased translation of this same protein in YL15-S, rhythmicity is restored (Figure 4B). This conclusion is consistent with data reported elsewhere (Merrow et al., 1997) showing that at least 10 molecules of FRQ per nucleus are needed at 25°C to complete the negative feedback of FRQ on the amount of its transcript. Recent genetic data also support this conclusion: a novel *frq* allele, frq^{11}, identified in a screen for mutations resulting in loss of rhythmicity at 30°C (Nakashima and Onai, 1996), is due to a 4 nt insertion between the AUG#2 and AUG#3. This makes the FRQ translational context similar to that of the YL34-S, and its circadian phenotype (loss of rhythm at high temperature) is just what would be expected based on the phenotype of YL34-S (Figure 2). It is not immediately clear why production of a single form of FRQ could not be regulated so as to produce amounts sufficient for the rhythm at all temperatures, although part of the answer may be that too much FRQ is as bad as too little and that normal temperature controls on translation would not allow a dynamic range sufficient for the required FRQ production at all temperatures.

The case for qualitative differences in the two FRQ forms is also strong; the data clearly show that while either form of FRQ can support a functional clock at intermediate temperatures, specific forms appear necessary for wild-type rhythmic control at physiological temperature extremes. For instance, at 18°C the FRQ levels in YL34-S and JC101-L are similar (Figure 4), but JC101-L is arrhythmic while YL34-S is rhythmic.

A final rationale for the requirement for multiple FRQ forms could reflect requirements for circadian output as distinct from assembly of the oscillator. Some component(s) of the clock must serve not only in executing the feedback loop comprising the clock but also in initiating output pathways from the clock. It may be that FRQ also plays a direct role in output and that a part of the deterioration in the overt rhythm we see in strains having only one form of FRQ could be the result of poor regulation of output, despite the assembly of a perfectly functional oscillator.

What Is the Mechanism of the Temperature-Regulated Translational Control of FRQ?

In eukaryotic cells, choice of mRNA-translation start sites is generally thought to be controlled by a ribosomal-scanning mechanism (Kozak, 1992)—the 40S ribosomal subunit first binds the mRNA 5′ cap structure

and scans along the mRNA in a 5′ to 3′ direction, becoming associated with eIF2.GTP.Met-tRNA$_i$ in a ternary complex competent to recognize the initiation codon. Upon encountering the first initiation codon in a favorable context, the 60S subunit joins the scanning complex to form the 80S ribosome and to initiate protein synthesis (Hinnebusch, 1996; Jackson, 1996). Data from a variety of systems suggest that regulation of translation is chiefly at the level of initiation, although *trans*-acting factors and *cis*-acting elements (the secondary structure of mRNA, the sequence context around the initiation codon, internal ribosome entry sites, and short open reading frames in the 5′ untranslated region of a transcript [uORFs in the 5′ UTR]), can influence the choice of start site and the amount of protein made (Jackson, 1996). In control of *GCN4*, for example, phosphorylation of eIF2 is influenced by availability of amino acids, which determines whether initiation occurs at a uORF or at the GCN4 ORF and thereby sets the amount of GCN4 made (Hinnebusch, 1996). Translational regulation of heat shock protein synthesis is also influenced by temperature-dependent phosphorylation of eIF2 (Duncan, 1996), providing a precedent for temperature effects on translation initiation.

Ambient temperature determines the total amount of FRQ translated and the ratio of FRQ$^{1–989}$ versus FRQ$^{100–989}$ in the cell. Both aspects of regulation appear to be mediated by temperature-dependent effects on the choice of AUG, regulation that may derive from interactions between the 40S ternary initiation complex, potential *cis*-acting elements in the transcript, the 6 uORFs in the 1.5 kb 5′ UTR of the *frq* mRNA (the last of which ends just 37 nt from FRQ AUG#1), and the 2 in-frame initiation codons used to make FRQ. The data are consistent with the possibility that alternative initiation of FRQ is due to a leaky scanning mechanism: inactive 40S ribosomes might enter the *frq* mRNA upstream of AUG#1 by either 5′ cap-dependent or -independent mechanisms and scan downstream initiating translation at AUG#1 or AUG#3. Temperature could affect the choice of FRQ AUGs by determining the rate at which the active ternary initiation complex forms upstream of AUG#1 (Figure 7). In this model, at higher temperatures active 40S ribosome ternary complexes form rapidly and initiate FRQ translation at AUG#1. In contrast, at low temperature the ribosomes require a longer scanning distance to bind to the eIF2.GTP.Met-tRNA$_i$ complex, so that more ribosomes fail to initiate at AUG#1 and instead initiate at AUG#3. This scenario could accommodate the regulation seen here but would not preclude the existence of temperature-sensitive *cis*-acting elements within the 5′ UTR of FRQ.

Temperature is one of the most pervasive clock-affecting agents known. We have determined that the amount and ratio of FRQ forms in the cell are a direct reflection of the ambient temperature. The existence and regulation of the two forms of FRQ begin to provide an explanation for how the physiological temperature limits permissive for rhythmicity are established and for how abrupt temperature changes might reset the clock. Furthermore, they demonstrate that temperature compensation of circadian period length at least in this system is a dynamic process that embraces a substantial temperature-dependent change in the amount of a clock element rather than being a homeostatic mechanism that holds the level of FRQ constant over a range of temperatures. Along with previous work describing the mechanism for light-induced resetting of this circadian clock (Crosthwaite et al., 1995), this study provides a view of the interface of a circadian system with its environment, a view where the Neurospora circadian clock can be seen as a well-balanced dynamic apparatus that is sensitive to environmental signals while remaining robust, compensated, and well tuned in their absence.

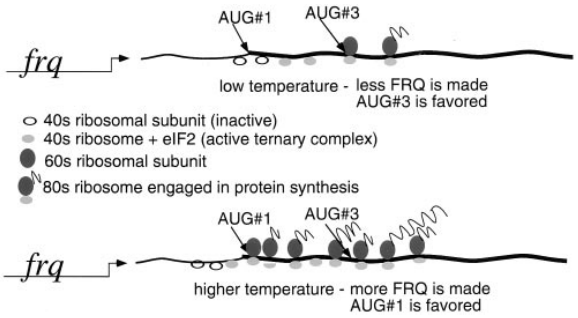

Figure 7. Translational Control Dictates the Amount and Forms of FRQ Synthesized and Thereby Sets the Physiological Temperature Range for Rhythmicity

The straight line and arrow on the left signify the *frq* promoter, and the wavy line signifies the *frq* transcript, the thicker portion of which represents the position of the long ORF encoding both forms of FRQ. 40S ribosomal subunits bind to the transcript upstream of AUG#1 and scan down, forming active ternary complexes with charged eIF2 and active 80S ribosomes upon encountering an AUG in a favorable context. Ambient temperature dictates the absolute size of the FRQ pool and the relative contributions of FRQ$^{1–989}$ versus FRQ$^{100–989}$ by influencing the relative efficiencies with which AUG#1 and AUG#3 are used. See text for details.

Experimental Procedures

Strains, Growth Conditions, and Race Tube Assay

For rhythmic experiments, Neurospora strains 30–7 (*bd*; *frq*$^{+}$*A*) and 93–4 (*bd*; *frq*^{10}*A*; *his-3*) (Aronson et al., 1994b) were cultured as described previously (Aronson et al., 1994a; Crosthwaite et al., 1995). All samples for protein extraction were harvested at CT8, the peak of FRQ expression, unless specified. When used, cycloheximide was added to cultures to reach final concentration of 10 μg/ml after the cultures were grown in constant light at 18°C or 30°C for at least 18 hr. All the harvested cultures were quick frozen in liquid nitrogen and stored in −80°C until protein extraction.

Race tube assay medium contains 1× Vogel's, 0.1% glucose, 0.17% arginine, and 50 ng/ml biotin. Densitometric analyses of race tubes and calculations of period length and phase of the rhythm were done using the Chrono II version 9.3 (Dr. Till Roenneberg, Ludwigs-Maximillian University, Munich).

Plasmids and Neurospora Transformation

pKAJ120, which contains the whole *frq* locus and *his-3* targeting sequence, was the parental plasmid for all other *frq* constructs (Aronson et al., 1994b). The construction of pYL15-S, YL31-N, and pJC101-L was described elsewhere (Garceau et al., 1997). In brief, pYL15-S has a DraIII-SphI deletion in pKAJ120 deleting AUG#1, AUG#2, and a large portion of the 5′ UTR. pJC101-L has a point mutation (AUG to GAU) at AUG#3. pYL34-S has a 4 nt deletion at the SphI site, eliminating AUG#2 and leaving AUG#3 as the only initiation codon for FRQ. Although AUG#1 is still available, its translational product is short and out of frame from the FRQ ORF. pYL31-N combines the mutations of pJC101-L and pYL34-S. pYL47 and

pYL48 were constructed using the Transformer Site-Directed Mutagenesis kit from Clontech Laboratories, Inc. (Palo Alto, CA). An *frq* HindIII fragment containing all the 5′ UTR and all the AUGs from pKAJ120 was cloned into pUC19, and the resulting plasmid used as the in vitro mutagenic template. The mutagenic primer for making AUG#1 deletion is GATAGGGTGAAC**ATT**GCGGATAGTGGGGA, which changed ATG#1 to ATT. To introduce a single nucleotide deletion between AUG#1 and AUG#2, the mutagenic primer GTGAACATGGC GG**AA**GTGGGATAAATC was used. The selection primer Trans Oligo NdeI/NcoI from the kit was used in both reactions. The SphI-MluI fragments from the resulting constructs were then inserted into SphI-MluI-digested pKAJ120 to create pYL47 and pYL48 so that AUG#1 is deleted in pYL47 and is out of frame from the FRQ ORF in pYL48. All the constructs were confirmed by DNA sequencing.

All constructs were transformed into the *his-3* locus of strain 93–4 as previously described (Bell-Pedersen et al., 1996b).

Protein Analysis

Proteins were extracted, quantified, and Western blotted as described in the accompanying paper (Garceau et al., 1997). After the blot was developed by chemiluminescence (ECL, Amersham), the membranes were stained by 0.1% amido black, 45% methanol, and 10% acetic acids to verify equal loading of protein. X-ray films of Western blots were scanned, and densitometry was performed using the NIH Image 1.59. For normalization in densitometric analyses, total loading was gauged by scanning and integrating the stained protein signal from a wide region of the gel containing a number of time-invariant protein bands.

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